

Molecular Identification and Sequence Analysis of *Tobacco Leaf Curl Begomovirus* from Jember, East Java, Indonesia

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Begomovirus had been proved as the causal agent of leaf curl disease in tobacco in Indonesia, or commonly in Indonesia called as *penyakit krupuk tembakau*. Association of *Begomovirus* with the disease was further confirmed by sequence analysis. Amplification of the virus was conducted following whitefly (*Bemisia tabaci* Genn.) transmission. Fragment of DNA 1.6 kb was amplified by polymerase chain reaction (PCR) located within the replication initiator protein gene and coat protein gene (top region). Conserved sequence of stem loop region was found, included nonanucleotide sequence TAATATTAC present in all geminiviruses. *Begomovirus* associated with leaf curl disease in tobacco showed the closest relationship with *Ageratum yellow vein virus - Zimbabwe*, a strain of *Tobacco leaf curl virus* from Southern Africa. It was also known that *Begomovirus* associated with leaf curl disease in tobacco from Jember, East Java was different from other Indonesian *Begomoviruses* reported earlier.

Key words: *Begomovirus*, leaf curl disease

INTRODUCTION

Geminivirus is a group of plant viruses with a distinct morphological characters. Its twinned isometric particles consists of circular single-stranded (ss) DNA genomes (Bock 1982). They are classified into four genera, i.e. *Mastrevirus*, *Curtovirus*, *Begomovirus*, and *Topocuvirus*, based on their vector relationship, host range and genome organization (Van Rogenmortel 2000). Members of the genus *Begomovirus* are transmitted by the whitefly *Bemisia tabaci* Genn. (Hemiptera: Aleyrodidae) and infect dicotyledonous plants. Diseases which were caused by whitefly-transmitted geminiviruses (WTG) have become a serious constraints to crops in tropical and subtropical areas throughout the world (Idris & Brown 1998; Samretwanich *et al.* 2000). In the last five years, geminivirus has been reported to cause significant yield loss in chilli pepper and tomato in vegetable growing areas in Java (Hidayat, unpublished data). However, tobacco leaf curl disease caused by geminivirus in Indonesia was reported earlier by Thung in 1932 (Trisusilowati *et al.* 1990). That was probably the first report on geminivirus infection in Indonesia. In 1984, *Tobacco leaf curl virus* (TLCV) caused serious damage in Bojonegoro, East Java, with up to 30% disease incidence (Poerbokoesoemo 1984 in Trisusilowati *et al.* 1990). The symptoms of TLCV infection include leaf curling, vein banding, uneven leaf surface, and rigid leaves. The disease may effect not only the yield but also the quality of tobacco leaf, especially when the leaves were targeted for cigar wrapping.

Observation conducted by Trisusilowati *et al.* (1990) using electron microscope demonstrated that a unique twin isometric particle was associated with leaf curl disease in tobacco. They

were also proved that the virus was transmitted through whitefly, *B. tabaci*. Furthermore, Aidawati *et al.* (2002) carried out a transmission study to elucidate the characteristics of TLCV transmission by its vector, *B. tabaci*. A single whitefly was able to transmit the virus and the efficiency of transmission were increased when the number of adult whiteflies was increased up to 20 per plant. Inoculation access period of 1 h could cause transmission up to 20% and the optimum inoculation access period was 12 h. Acquisition access period of 30 min resulted in 70% transmission while 100% transmission occurred with a 24 h-acquisition access period. The virus was proven to be persistently but not transovarially transmitted.

The evidence above has confirmed the association of geminivirus in tobacco leaf curl disease in East Java. Later on, polymerase chain reaction (PCR) using specific degenerate primers for geminivirus, PAL1v1978, and PAR1c715, was successfully amplified a 1.6 kb DNA fragment from infected tobacco plants as well as viruliferous whiteflies (Aidawati *et al.* 2002).

In this paper we reported our attempt to clone and sequence the geminivirus causing tobacco leaf curl in Jember, East Java. Its relationship with other geminiviruses, especially those reported from Indonesia and those causing leaf curl disease on tobacco from other part of the world, was analysed based on nucleotide sequence homology.

MATERIALS AND METHODS

Collection and Maintenance of Virus Isolate. Leaves showing TLCV symptom were collected from tobacco field at Arjasa and Tegal Gede villages, Jember, East Java. The virus was maintained on tobacco plants (*Nicotiana tabacum*) H382 by insect transmission (Aidawati *et al.* 2002). Adults *B. tabaci*

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were obtained from broccoli plants in Bogor and identified using identification key of Martin (1987). The insect were reared on tobacco and broccoli (*Brassica oleraceae* var. *italica*) plants in whitefly-proof cages. Tobacco plants for maintaining virus isolates were grown in a whitefly-proof screenhouse.

PCR-Based Detection Using Geminivirus Degenerate Primers. DNA template for PCR was prepared from infected tobacco plants following method developed by Dellaporta *et al.* (1983). The DNA pellet was resuspended in 50 µl of sterile distilled water. Amplification of geminivirus genome was proceeded using a pair of degenerate primers designed for the amplification of the DNA A genomic component, pAL1v 1978 (5'GCATCTGCAGGCCACATYGTCTTYCCNGT3') and pAR1c 715 (5'GATTTCTGCAGTTDATRTTYTCRTCCAT CCA3') (Rojas *et al.* 1993). Amplification with PCR technique was carried out in a 25 µl reaction mixture containing 1 µl of sample DNA solution and 0.2 µM of each primer using Ready To Go PCR kit (Amersham Life Science). PCR was performed in thermocycler Gen Amp PCR System9700 (Perkin Elmer) with 30 cycles of melting, annealing and DNA extension at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min, respectively. PCR products were then analysed by electrophoresis in 1% agarose gels in Tris-buffer EDTA.

Cloning and Sequencing of DNA from TLCV Isolate. DNA fragments of approximately 1.6 kbp, as a product of PCR amplification, was cloned into pGEM- T Easy vector (Biorad) with *Pst*I cloning site. Selected DNA clone was then sequenced by the dideoxy nucleotide chain termination method (Maniatis *et al.* 1982). A 1.6 kbp viral DNA fragment was completely sequenced on both strands using internal primers that was designed based on the prior nucleotide sequences.

Once the sequence was completed, it was compared with those of other whitefly-transmitted geminiviruses available in Genbank (Table 1) using Clustal W program version 1.82 European Bioinformatics Institute (EMBL-EBI: www.ebi.ac.uk/serve/clustalW). Phylogenetic analyses was conducted with PAUP program version 4.0 b4a using Maximum-Parsimony method with heuristic searches using the TBR branch swapping option and 10,000 random addition sequences.

RESULTS

Symptoms of TLCV-Infected Tobacco Plants. Whitefly transmission of TLCV to tobacco plants was conducted following a procedure established by Aidawati *et al.* (2002). Whiteflies were given a 24 h acquisition feeding period on virus source before transferred to healthy tobacco for a 48 h inoculation feeding period. In general, the symptoms of TLCV on tobacco plants (*N. tabacum*) H382 was developed 7-10 days after inoculation feeding period. Infected plants will show leaf malformation involving upward curling and vein banding with crinkle-like symptom (Figure 1).

Identification of TLCV by PCR and Sequence Characterization. Specific DNA fragments of 1,600 bp was successfully amplified from infected tobacco plants using geminivirus-specific degenerate primers pAL1v 1978 and pAR1c 715. The amplified DNA fragment, denoted as “top

region”, include part of replicase region, full common region, and part of coat proteion region. The PCR product was then cloned into the PGEM-T easy vector. Following screening of recombinant DNA, a clone identified as pTobT8 was selected for viral sequence analyses. Nucleotide sequence of TLCV (pTobT8) from base 1 to 1474 was determined and submitted to Genbank (Accession No. AB246171). The nucleotide sequence of 33 base stem-loop region was found in the sequence of pTobT8 as well as the conserved nonanucleotide sequence TAATATTAC which is known as TATA-box region (Figure 2). Both stem-loop and TATA-box regions has been found in all geminiviruses sequenced so far (Ikegami *et al.* 1988), as it was observed in other sequences of begomoviruses from Indonesia. The isolate of *Begomovirus* associated with leaf curl disease of tobacco in Jember, East Java was tentatively denoted as *Tobacco leaf curl Indonesia virus-Jember* (TLCIV- Jbr).

Phylogenetic Analyses of TLCIV-Jbr. Relationship between TLCIV-Jbr and other selected begomoviruses was evaluated based on “top region” sequences. Although conserved sequence of the geminivirus’s common region was observed, it was found that TLCIV-Jbr was different with the other geminiviruses that has been reported from Indonesia, i.e those infecting chillipepper, tomato, and weed (*Ageratum conyzoides*). Analyses of sequence identity using Maximum-Parsimony method furthermore revealed that TLCIV-Jbr was distinct from any other tobacco leaf curl viruses (Figure 3).

DISCUSSION

A geminivirus was first demonstrated to be the causative agent of tobacco leaf curl disease in Indonesia in 1990. However, the disease has been reported earlier by Thung in 1932 and caused serious problem in tobacco plantation in East Java (Trisusilowati *et al.* 1990). Infection of TLCV may cause various symptoms including leaf curling, vein banding, uneven leaf surface, and rigid leaves (Aidawati *et al.* 2002). Therefore, in Indonesia the disease is commonly called *penyakit kerupuk*. Variation on disease symptom developed from TLCV infection has also been documented. A perplexing feature of tobacco leaf curl since its earliest reports has been the observed variation in symptom severity. For instance in Southern Africa, at least three symptom phenotypes in tobacco have been identified that could be classified as

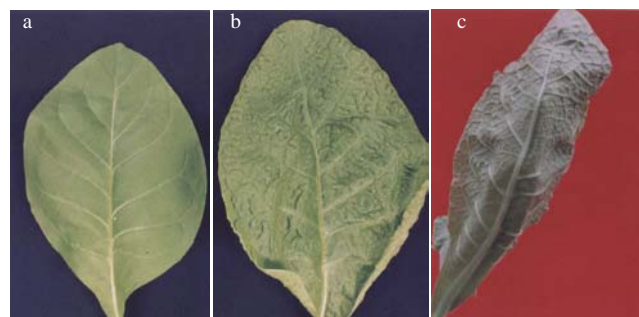


Figure 1. Symptom of *Tobacco leaf curl virus* on tobacco plants (*N. tabacum*) H382. a. Healthy leaf, b. Leaf showing mild symptom, c. Leaf showing severe symptom.

TLCIV-Jbr	TTTGGGAGGTGCCATTTGACTTGGTCAATCGGGTCTCAACAACTTGCCTTAGCAATCGG 60
AYVV-LBII	TTTGGGAGGAACCATT-GACTTGGTCAATTGGAGACATTCATAGTT-CTCTGTCAATTGG 58
ToLCJAV-DM	TTTGGGAGGAGCCATT-GACTTGGTCAATTGGAGACATTCATAGTT-CTCTGTCAATTGG 58
PYLCIV-Bgr	ATTGGAGTGTGCTTTTGGATTGGGACAATCACTTCTATCCCTATGT-----ATTGG 51
PYLCIV-LBI	ATTGGAGTGTGCTTTTGGATTGGGACAATCACTTCTATCCCTATGT-----ATTGG 51
PYLCIV-LBII	ATTGGAGTGTGCTTTTGAATTGGACAATCACTTCTATCCCGTAT-----ATTGG 51
	* * * * * * * * * * * * * * * * * * * *
TLCIV-Jbr	GGAATGGGTCCCAATATATAGTGAGGACCTAAATGGCATTATTGTAATTCTCGAAAGTAA 120
AYVV-LBII	AGA-CAGGAGACAATATATAG-GTGTCTCCAAATGGCATAATTGTAATTTCTACAAG-AA 115
ToLCJAV-DM	AGA-CAGGAGACAATATATAG-GTGTCTCCAAATGGCATAAGTTCGTAATTTCTCATAA-TA 115
PYLCIV-Bgr	AGA-CAGGAGACAATATATAT-AAGTCTTATAATGGCTTTTAG-TAATTTTGTACAC-CA 107
PYLCIV-LBI	AGA-CAGGAGACAATATATAT-AAGTCTTATAATGGCTTTTAG-TAATTTTGTACAC-CC 107
PYLCIV-LBII	AGA-CAGGAGACAATATATAT-AAGTCTTATA-TGGCTTTTAGGTAATTTTGTACAC-CC 107
	* * * * * * * * * * * * * * * * * * * *
TLCIV-Jbr	TTCAAATTTCAAATTTTGAATCCAATAGCGGCC-ATCCGTATAATATTACCGGATGGC-C 178
AYVV-LBII	TTCAAT-----TCCAAAAGCGGCC-ATCCGTA-AATATTACCGGATGGC-C 158
ToLCJAV-DM	TTTAA-----TTTGAAATCAGCGGATCCGTA-AATATTACCGGATGGC-C 158
PYLCIV-Bgr	TTGAAT-----GGTTAAAGCGGC--ACTCGTATAATATTACCGAGTGCCGC 151
PYLCIV-LBI	TTGAAT-----GGTTAAAGCGGC--ACTCGTATAATATTACCGAGTGCCGC 151
PYLCIV-LBII	TTGAAT-----GGTTAACCACGC--GCTCGTATAATATTACCGAGTGCCGC 151
	* * * * * * * * * * * * * * * * * * * *
TLCIV-Jbr	GCGATTTTTTTTAAAGTGGTCCCCCCCCACTAACAAATGTCCCCCAATC----- 225
AYVV-LBII	G-GATTTTTTTTAAAGTGGTCCCCACCCTAA-AAATGTCCCCCACTCATAACGCTCCCTC 216
ToLCJAV-DM	G-GATTTTTTTTAAAGTGGTCCCTCCCTCCTAAAGAAATGTCCCCCAATCAGAACGCGCCCTC 217
PYLCIV-Bgr	GAAAATATTTAAATGTGGTCCCCCAAGCCACTTTTGACTGACCAATCACGTTGTATCACA 211
PYLCIV-LBI	GAAAATTTTAAATGTGGTCCCACAAGCCACTTTTGACTGACCAATCACGTTGTATCACA 211
PYLCIV-LBII	GAAATTTTTTAAATGTGGTCCCACAAGCCACTTTTGACTGACCAATCACGTTGTATCAG 211
	* * * * * * * * * * * * * * * * * * * *
TLCIV-Jbr	-----
AYVV-LBII	AAAGCTTAT----- 225
ToLCJAV-DM	AAAGCTAA----- 225
PYLCIV-Bgr	AAGCTTGTTAGTGC- 225
PYLCIV-LBI	AAGCTTGTTAGTGC- 225
PYLCIV-LBII	AAGCTTGGT-GTACC 225

Figure 2. Alignment of nucleotide sequences of the common region of *Tobacco leaf curl Indonesia virus-Jember* (TLCIV-Jbr) with other reported Indonesia begomoviruses as listed in Table 1. The alignment showing TATA sequences (underlined sequences) and the stem-loop region (bold letters for TLCIV-Jbr).

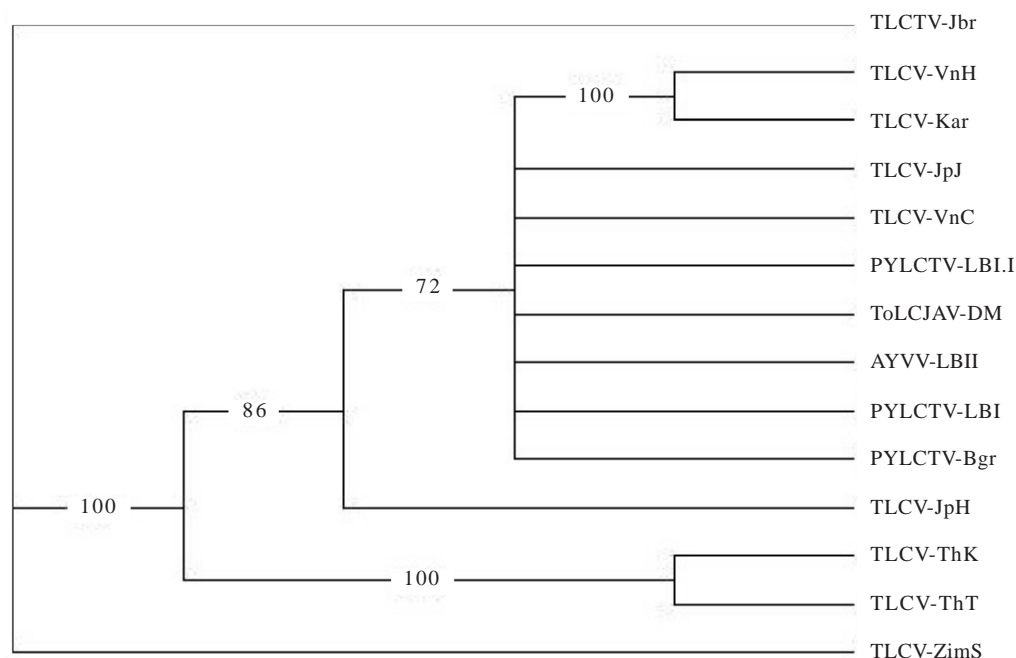


Figure 3. Phylogenetic tree based on the alignments of nucleotide sequences of "top region" of *Tobacco leaf curl Indonesia virus-Jember* (TLCIV-Jbr) with other begomoviruses as listed in Table 1.

Table 1. List of Begomoviruses used for viral sequence analysis

Genbank accession number	Organism	Nucleotide length (bp)	Geography origin	Host plant	Acronym
AB189845	<i>Pepper yellow leaf curl Indonesia virus</i>	1563	Indonesia: Lembang, West Java	<i>Lycopersicon esculentum</i>	PYLCIV-LBI
AB246170	<i>Pepper yellow leaf curl Indonesia virus</i>	1491	Indonesia: Segunung, West Java	<i>Capsicum frutescens</i>	PYLCIV-Bgr
AB189913	<i>Ageratum yellow vein virus-Indonesia</i>	1557	Indonesia: Lembang, West Java	<i>Ageratum conyzoides</i>	AYVV-LBII
AB189848	<i>Tomato leaf curl java virus-Magelang</i>	1562	Indonesia: Magelang, Central Java	<i>L. esculentum</i>	TolCJAV-DM
IB189850	<i>Pepper yellow leaf curl Indonesia virus</i>	1555	Indonesia: Lembang, West Java	<i>C. annuum</i>	PYLCIV-LBII
AM051086	<i>Tobacco leaf curl virus-[Vietnam]</i>	526	Vietnam: Cao Bang Province	<i>N. tabacum</i>	TLCV-VnC
AM051085	<i>Tobacco leaf curl virus-[Vietnam]</i>	526	Vietnam: Hang Tay Province	<i>N. tabacum</i>	TLCV-VnH
AY007616	<i>Tobacco leaf curl virus-[India]</i>	771	India: Karnataka	Tobacco	TLCV-Kar
AY633751	<i>Tobacco leaf curl virus-[Thailand]</i>	771	Thailand: Tak Province	Tobacco	TLCV-ThT
AY633750	<i>Tobacco leaf curl virus-[Thailand]</i>	771	Thailand: Kamphaensaen	Tobacco	TLCV-ThK
AB108838	<i>Tobacco leaf curl virus-[Japan]</i>	1570	Japan: Kagoshima	Honeysuckle	TLCV-JpH
E15418	<i>Tobacco leaf curl virus-[Japan]</i>	2766	Japan	<i>Eupatorium japonicum</i>	TLCV-JpJ
AF077749	<i>Tobacco leaf curl virus-[Japan]</i>	777	Zimbabwe	<i>Ageratum conyzoides</i>	TLCV-ZimS

tobacco leaf curl. Based on sequence analyses, different virus strains are unlikely to be found in the infected plants (Paximadis & Rey 1997). The discovery of defective DNA molecules in leaf curl-affected tobacco plants may possibly explain the variable symptom severity. The environmental factors and type of tobacco varieties may also contribute to symptom severity. Agroinoculation study using a full-length genomic DNA –A derived from a field tobacco plant exhibiting mild symptoms in *N. tabacum*, but *N. benthamiana* did exhibit severe leaf curl symptoms (Paximadis & Rey 2001). The possibility of satellite virus similar to DNA β associated with *Ageratum yellow vein virus* from Singapore (Saunders *et al.* 2000), *Cotton leaf curl virus* from Pakistan (Briddon *et al.* 2001), or *Tomato leaf curl Java virus* from Indonesia (Kon *et al.* 2006) might contribute to symptom severity. Therefore it is also interesting to determine whether TLCIV-Jbr infection is associated with the presence of its DNA β .

Begomoviruses has been reported to infect tomato, chilli pepper, and *A. conyzoides* in Indonesia and it was reported that tobacco plant, *N. tabacum*, is one of the host plants of all those begomoviruses (Rusli *et al.* 1999; Aidawati *et al.* 2002; Hidayat *et al.* 2002; Haerani & Hidayat 2003; Sulandari *et al.* 2006). Conserved sequence in the common region was observed for all Indonesia begomoviruses (Figure 2); however, the phylogenetic analysis revealed that TLCIV-Jbr was grouped in different cluster from those of other *Begomovirus* isolates reported from Indonesia (Figure 3). We understand from previous sequence analysis that *Begomovirus* species from Indonesia, *Tomato leaf curl Indonesia virus* (TolCIDV), *Tomato leaf curl Java virus* (TolCJAV), *Ageratum yellow vein virus-Java* (AYVV-[Java]), PYLCIV-Bgr, had close relationship one to each other (Sukanto *et al.* 2005; Kon *et al.* 2006; Hidayat

et al. 2006). The fact that TLCIV-Jbr was not clustered with the other Indonesian begomoviruses might indicated that TLCIV-Jbr was possibly different *Begomovirus* species. Furthermore, when analysis was conducted using nucleotide sequences of other geminiviruses causing leaf curl disease on tobacco, TLCIV-Jbr was clustered only with TLCV-Zims (Figure 3). This facts strengthened the hypotheses that there was a high degree of genetic diversity among *Begomovirus* group, which might be emerged due to a high degree of pre-existing genetic diversity among begomoviruses or possible recombination between different virus species during infection (Ribeiro *et al.* 2003). The knowledge regarding genetic diversity among members of geminivirus infecting different crops and weeds in Indonesia should be considered in developing disease control strategies.

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